Countercurrent Distribution Studies of Adrenocortical Steroids and Steroidal Substances in the Aortic and Adrenal Vein Blood of Anaesthetized Dogs

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Introduction

This survey is based on the following publications which will be referred to in the text by their roman numerals:

I. Hans Carstensen, Countercurrent distribution studies of adrenocortical steroids. *Acta. Soc. Med. Upsal. 61,* 26 (1956).

II. Hans Carstensen, Use of countercurrent distribution for the identification of aldosterone (Δ^4-pregnene-21-ol-3,20-dione-18-al). *Arkiv för Kemi 10,* 235 (1956).

III. Hans Carstensen, Countercurrent distribution studies of steroidal substances in dog aortic and adrenal vein blood. *Acta Soc. Med. Upsal. 61,* 137 (1956).

The object of the present study was to elaborate methods of separation of the adrenocortical steroids by countercurrent distribution and to determine their partition coefficients in various solvent systems (papers I and II). The methods were applied to steroidal compounds in the adrenal venous effluent of anaesthetized dogs (paper III).

1. Model experiments on steroids

The partition coefficient, K, of a substance, i.e. its distribution ratio between equal volumes of a pair of immiscible solvents at a certain temperature ($K = \mathrm{conc}_{\text{upper phase}}/\mathrm{conc}_{\text{lower phase}}$), is a physical constant under ideal conditions (dilute solutions). The partition coefficient as other physical constants may be used for the characterization and identification of a substance. If several substances occur together the determination of the partition coefficient is not possible by such simple means as the distribution between immiscible solvents in a single vessel (separating funnel).

For this purpose it is necessary to achieve a separation of the substances by repeated distributions into new batches of solvent. However, the use of separating funnels for this purpose is arduous as both phases have to be repeatedly extracted with new solvent of the other phase. Such a distribution may be carried out in a countercurrent way using the technique developed by Craig (1). For analytical purposes in order to determine the values of the partition coefficients a partial separation which reveals the maximum concentration of the solutes in different parts of the apparatus is sufficient. The distribution of the solute into the tubes may be expressed in terms of a binomial expansion $(a + b)^n$, where a is the fraction of the total quantity which in each tube (in so far as K is constant) is contained in the lower phase ($a = 1/(K + 1)$), b is

the fraction contained in the upper phase ($b = K/(K+1)$), and n is the number of transfers. If for example two transfers have been carried out [$(a+b)^2 = a^2 + 2ab + b^2$] it is easily seen that (1) if $K = 1$, $a = b = 1$ and the maximum concentration will be contained in the middle tube; (2) if $K < 1$, $a > b$ and the maximum concentration of the solute will be found in the first two tubes of the apparatus (the first terms in the expansion which contain a); (3) if $K > 1$, $b > a$ and the maximum concentration is in the last two tubes of the apparatus (the last terms in the expansion which contain b). The theoretical curves thus constructed from the binomial expansion are not symmetrical except when $K = 1$. In a few cases (III) the greater quantity of material made it desirable to load several tubes with equal amounts before the start of the distribution procedure in order to avoid too concentrated solutions. This is essentially the performance of several countercurrent distributions in parallel (2). As the theoretical curves are not symmetrical it follows that there is a shift of the position of the maximum concentration for the composite curve (except in the case of $K = 1$).

It is possible to separate two substances in a certain solvent system only if the values of the partition coefficients differ. The efficiency of a solvent system increases with the ratio of higher to lower K, β. A great part of the present work is therefore the elaboration of suitable solvent systems for the separation of the steroids in question (I). This was attempted by varying the relative concentrations of the phase components systematically and plotting in a diagram the K values of a substance against the relative concentration of a phase component.

The basis for the arrangement of the K values within different solvent systems in the case of steroids rests in the number of carbon atoms of the side chain at C_{17} (if any), angular methyl groups, and the number, position and steric arrangement of hydroxyl and keto groups. It was generally found that the corticosteroids and their related steroids investigated were arranged in the same order within most solvent systems. One exception to this was found within systems containing 2-butanol, n-hexane and water. The difference in sequence was the following (I, II):

"typical sequence"	*"atypical sequence"*
cortisol	aldosterone
cortisone	cortisol
aldosterone	cortisone
corticosterone	11-ketocortexone
17-hydroxycortexone	corticosterone
11-ketocortexone	17-hydroxycortexone
cortexone	

The atypical sequence offered better separation of 17-hydroxycortexone/11-ketocortexone and of aldosterone/cortisone than did other solvent systems.

The elaboration of relatively efficient solvent systems was essential in the present investigation (III) as the facilities available limited the maximal number of transfers to be made (in the general procedure). The open type unit used may also limit the number of transfers in a distribution procedure as the evaporation of solvents may shift the phase composition seriously if a large number of transfers are to be made. This will be an experimental error even in using a moderate number of transfers especially for multicomponent systems. A closed type of apparatus might have reduced the variations of the K values sometimes seen (e.g. I; within group *I 30*).

The maximal separation factors (β) for pairs of closely related corticosteroids were within the range 2.9 (corticosterone/11-ketocortexone)to 59 (11-ketocortexone/cortexone) (I: *Table 3*). As a rule duplicate determinations were carried out, using only one steroid in each experiment and performing two experiments simultaneously ($n = 24$). In critical cases U.S.P. reference standard steroids were investigated to insure that a similar result was obtained on the standardized substances.

The inclusion of some 17-keto steroids (in some cases prepared by bismuthate oxidation) and of some acetylation products in (I) is of minor importance in the present work as the formation of oxidation products and acetates were not used in (III) for identification purposes. The reason for this was that the methods employed for oxidation and acetylation gave variable results with regard to the recovery and in the case of the bismuthate oxidation products apparently also with regard to the nature of the oxidation products formed (I). It is hoped that these difficulties can be overcome in future work.

2. Use of countercurrent distribution characteristics for the elaboration of methods for steroid extraction (III)

The main problem encountered in the extraction of steroids from natural sources was the separation from contaminating materials. This is somewhat critical in the case of the corticosteroids which because of their relatively high water solubility are difficult to separate from other water soluble substances. The choice of organic solvent is therefore very important. Experiments carried out with ethyl acetate showed (I) that some polar steroids investigated favoured the ethyl acetate phase while the bulk of lipids was contained in the water phase. After ethyl acetate extraction, re-extraction of blood with chloroform or ethyl acetate showed practically no polar steroid group in subsequent countercurrent distributions. Similar results using other techniques have been reported in the litterature.

These statements may be valid for the extraction from whole blood and plasma though the higher solubility of corticosteroids in albumin solutions demonstrated by Eik-Nes (3) may favour the watery phase a little more than if the steroids were present in water only. In order to achieve better recovery, greater volumes of ethyl acetate were subsequently used in the first extraction and further this may reduce the interference of denatured and precipitated proteins. A "liquid spray extraction" method was worked out as it was desirable to increase the surface between blood (plasma) and extraction solvent without producing emulsions. The method is essentially the same as that later described by Levy and Kushinsky (4) and which in their hands was reported to give better recovery and less risk of producing artifacts than other methods which they tried.

3. *Applications of the methods to the quantitative estimation of steroidal substances in aortic and adrenal vein blood (III)*

(a) *Fractionation of the extracts*

The first experiments are to be regarded as exploratory approaches to the problem. It was found suitable to reduce the quantity of inert material prior to the countercurrent distributions. This was achieved by partition between *n*-hexane and 50 % aqueous ethanol as it could be assumed from the partition coefficients previously determined (I) that all corticosteroids should be recovered in the lower phase after two transfers. The upper phase contains the bulk of lipids.

The contents of this phase have been very little explored for adrenal cortical substances, possibly because it was found in the previous work upon adrenocortical extracts that they were less biologically active (5). However, after recovery of the ketonic fraction from Girard separation it is evident that at least one substance in this fraction was added by the adrenal to the blood.

In some other experiments in which a relatively large volume of pooled plasma was used, no previous fractionation was carried out. The polar fraction was recovered by a "preparatory" countercurrent distribution and then distributed using initial spread of the sample in 5 tubes. The non-polar fraction may have contained large quantities of lipids as a great number and quantity of different components appeared. This made it difficult to solve the problem of isolating certain corticosteroids.

(b) *Adrenal output of steroidal substances*

The evaluation of the output of any substance from the gland is treated in (III: A). It requires the determination of the flow rate of blood through the gland and assumes stationary conditions and disregards the lymph drainage. The simul-

taneous concentrations in aortic and adrenal vein blood were determined. Three such experiments are reported. Two of these were investigated by countercurrent distribution in more detail (311055 and the pooled plasma from 170554, 200554 and 240554). The extraction of plasma instead of whole blood may involve some losses as not all plasma can be recovered from the blood and no account was taken of the possibility that some steroids may be contained in the formed elements. The fraction not recovered undoubtedly is much smaller than the changes observed. It may, therefore, in this case, be valid to use the plasma flow rate to evaluate the output from the gland.

It is clear that such a quantitative evaluation of the result is only possible using a restricted number of subsequent countercurrent distributions. Not only varying losses encountered with each countercurrent distribution (as a rule in the order of 10–15 %) but also the risk of producing artifacts (during evaporation, by impurities not removed from the solvents, by degradation of the solvents e.g. phosgen from chloroform, etc.) are restricting factors.

From the analytical data, mainly employing the specific absorption of ultraviolet light at 240 $m\mu$ and the reduction of blue tetrazolium, a tentative conclusion was made that at least three substances were α,β-unsaturated ketones and 20, 21α-ketols. One very polar component was probably not an α,β-unsaturated ketone as the NaOH fluorescence reaction of Bush was negative. The same was probably true of another substance because of an atypical ultraviolet spectrum.

Preliminary output values for the major (from a quantitative point of view) corticosteroid, cortisol, were of the order of magnitude reported in the literature by others using chromatography. However, a subsequent resolution into at least three components (using systems containing 2-butanol and *n*-hexane) reduced the final output values by approximately 72 %. All components were probably derived from the adrenal. Three more polar reducing substances without specific ultraviolet absorption were also derived from the adrenal (total output: 3.9 μg cortisol equiv./kg/min). They are probably polyhydroxy steroids with an α-ketol side chain.

The output values of some other corticosteroids, corticosterone and 17-hydroxycortexone, were uncertain as the aortic and adrenal vein blood samples were not processed in the same way in the application of the countercurrent distributions. The concentration in the adrenal vein blood of corticosterone was lower than expected from the literature while that of 17-hydroxycortexone was of the expected order of magnitude.

For comparison a sample of arterial blood from an adrenalectomized dog was also investigated (C:c, v). The data were computed for the same volume of blood as in dog 311055. Their was a marked decrease of reducing substances in the ketonic non-polar fraction compared with both aortic and adrenal vein

blood of the intact dog. An additional component with specific extinction at 240 mμ was probably present as well as the major components found in the aortic blood. The substance which was found to be added by the adrenal in 311055 was probably entirely absent.

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